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Research report

Region-specific changes in NMDA receptor mRNA induced by chronic morphine treatment are prevented by the co-administration of the competitive NMDA receptor antagonist LY274614

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Abstract

The steady-state mRNA levels of the NMDA receptor NR1 subunit were determined by a quantitative solution hybridization assay in selected CNS regions associated with antinociception in the rat. Tissues were obtained by microdissection from rats treated chronically with morphine alone or in combination with LY274614, a competitive NMDA receptor antagonist. Morphine treatment for 7 days resulted in the development of tolerance to morphine's analgesic effect and produced a significant decrease in the steady-state NR1 mRNA levels in the spinal cord dorsal horn (by 16%), and an elevation in nucleus raphe magnus and medial thalamus (by 26 and 38%, respectively). The NR1 mRNA levels were unchanged in the lateral paragigantocellular nucleus, locus coeruleus, periaqueductal grey, and sensorimotor cortex. NMDA receptor binding in the spinal cord measured with [^{3}H]MK-801 was reduced approximately 50% by chronic morphine treatment. Co-administration of LY274614 (s.c. at 24 mg/kg/24 h via an osmotic pump) not only attenuated the development of morphine tolerance but also prevented the changes in the NR1 mRNA levels induced by chronic morphine administration. Neither a 7-day infusion of LY274614 nor an acute injection of morphine (10 mg/kg, s.c.) changed the NR1 mRNA levels. These results suggest that changes in the expression of the NR1 mRNA induced by chronic morphine in three CNS regions involved in antinociception are associated with the development of morphine tolerance and in the spinal cord, morphine tolerance is associated with the downregulation of NMDA receptors.
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Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Opioid receptors

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1. Introduction

Opioids are the most effective analgesics for treating many forms of acute and chronic pain [26]. However, the clinical utility of opioid analgesics is often hampered by the development of analgesic tolerance, which refers to a decreased potency of the drug and results in a need for progressively higher doses to achieve the desired effect [19]. Given that morphine remains the drug of first choice for the management of severe pain [20], the development of tolerance to its analgesic effect has broad clinical implications. The identification of the cellular and molecular events underlying morphine tolerance thus would not only contribute to our understanding of the effects of morphine and other opioids, but also point toward improved strategies for pain management.

A large body of data accumulated from our laboratory and others over the past decade suggest that the NMDA receptor, a subtype of glutamate receptor, plays a critical role in development of morphine tolerance (for review, see Refs. [19,24,36]). NMDA receptors are widely distributed in the mammalian CNS and are important in a variety of synaptic plasticity phenomena such as long-term potentiation (LTP) [30]. Numerous behavioral studies have shown that a wide variety of NMDA receptor antagonists, when co-administered with morphine, can prevent the develop-

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ment of tolerance to morphine's analgesic effects [24,25,35,37].

In contrast to the large number of behavioral studies examining NMDA receptor antagonists' capacity in preventing the development of morphine tolerance, little is known on how the opioid receptor and the NMDA receptor interact such that blockade of NMDA receptors by NMDA receptor antagonists results in attenuated morphine tolerance. NMDA receptor antagonists' effectiveness in preventing the development of morphine tolerance is not a direct interaction between these drugs with opioid receptors, as neither acute opioid analgesic effects nor the expression of opioid tolerance is affected by NMDA receptor antagonists [26,35,37]. Furthermore, chronic NMDA receptor antagonism does not change the affinity or density of opioid receptors [34]. It is believed that morphine treatment activates cellular kinases, including protein kinase C (PKC) and induces changes in the NMDA receptor complex, which in turn activate a variety of second messenger systems including Ca^{2+} and NO pathways [7,10,11], leading to the long lasting changes as seen in morphine tolerance [19,24]. Recent observations further suggest that chronic morphine treatment induces the downregulation of spinal glutamate transporters resulting in alterations in glutamate homeostasis [26].

In rats tolerant to systemic morphine, the binding of [^{3}H]MK-801 was decreased in midbrain and in spinal cord [4]. Chronic systemic morphine or MK-801 resulted in an increase in brain synaptic membrane glutamate binding [22]. In a model of spinal morphine tolerance resulting from the infusion of intrathecal morphine, no difference was observed in the binding of [^{3}H]MK-801 while the affinity was decreased [40]. A single dose of morphine had no effect on spinal cord levels of the mRNA for the NMDA receptor NR1 subunit, although changes were noted in hippocampus and hypothalamus [23]. Intracerebroventricular infusion of morphine results in increases in NR1 mRNA in locus coeruleus and hypothalamic paraventricular nucleus [45].

However, not all CNS regions associated with antinociception have been examined so far and it is not known whether NMDA receptor antagonists, when coadministered with morphine, would prevent these changes in gene expression as well as behavioral manifestations of morphine tolerance.

Therefore, using a quantitative solution hybridization technique [15], the present study examined the effects of chronic morphine on the expression of the mRNA encoding the NMDA receptor subunit NR1, which is required for a functional in vivo NMDA receptor [13], in a variety of CNS regions associated with morphine tolerance and nociception. We also examined NMDA receptor levels in spinal cord and the ability of a competitive NMDA receptor antagonist LY274614 to prevent changes in NR1 mRNA expression induced by chronic morphine.

2. Materials and methods

2.1. Subjects

All animal procedures were approved by the Institutional Animal Care and Use Committee (ICACU) of Weill Medical College of Cornell University. Adult male Sprague–Dawley rats weighting 250–300 g were purchased from Taconic Farms (Germantown, NY, USA). Animals were housed four per cage in a room maintained at 22–24 °C on a 12-h light/dark photocycle with light onset at 06:00 h. Food and water were available ad libitum.

2.2. Drugs

LY274614 [(±)-6-phosphonomethyl-decahydroisoquinolin-3-carboxylic acid] was generously provided by Dr Paul Ornstein (Eli Lilly Research Laboratories, Indianapolis, IN, USA). LY274614 was dissolved in sterile saline and the pH adjusted to 7.0 before transfer to the osmotic pump used to deliver this drug s.c.

Morphine sulphate for s.c. injection was obtained from Mallinckrodt (St. Louis, MO, USA). Pellet formulations for morphine and the corresponding placebo were obtained from the Research Triangle Institute (Research Triangle Park, NC, USA) through the National Institute on Drug Abuse (Rockville, MD, USA). Each morphine pellet contained 75 mg of morphine base. The placebo formulation omitted morphine, but was otherwise identical in composition to the morphine pellet.

2.3. Drug administration

LY274614 (24 mg/kg/24 h) was chronically administered by continuous s.c. infusion via an Alzet osmotic pump (model 2 ML-1) [35], surgically implanted into the subcutaneous space caudal to the dorsum of the neck, under halothane anesthesia, on the afternoon of day 0, 16 h before the implantation of morphine or placebo pellets on day 1 [34,35]. Control animals received sham surgery.

Morphine was administered chronically via the implantation of pellets (75 mg) [34]. Two morphine pellets wrapped in nylon mesh was surgically implanted into the subcutaneous space caudal to the dorsum of the neck, under halothane anesthesia on day 1 and two additional pellets were added on day 4. Controls were implanted with an equal number of wrapped placebo pellets.

2.4. Assessment of tolerance

The development of tolerance to morphine's analgesic effect was assessed on the morning of day 7 using a 52.5 °C hotplate (IITC, Life Sciences, Woodland Hills, CA, USA) surrounded by clear Plexiglas walls. The withdrawal latency from hotplate was measured from the placement of

the animal on the hotplate surface until the animal exhibited licking of either hindpaw or jumping behavior. An animal that failed to respond before 60 s (cut-off time) was removed from the hotplate and assigned a latency value of 60 s. A baseline latency was obtained prior to the injection of a challenge dose morphine (10 mg/kg) or saline [35]. This dose of morphine is an approximate ED90 dose as estimated from a dose–response analysis using the log probit procedure (Bliss) (see Fig. 2 of Ref. [34]). In a separate experiment, sham-operated, placebo-implanted animals received a challenge dose of morphine or an injection of saline. Subsequent response latencies were determined at 60 min.

2.5. Tissue collection and RNA extraction

In a separate group of animals, brain and spinal cord samples were collected for mRNA analysis. Following sacrifice by decapitation, rat brain (with cerebellum removed) and caudal spinal cord were cut into 1-mm thick coronal sections with the aid of a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, USA). The sections were transferred into ice-cold glass slides and inspected visually for the presence of selected landmarks from the atlas of Paxinos and Watson [31]. The seven areas dissected from rat brain and spinal cord for RNA extraction and analysis (Fig. 1) including the dorsal horn of spinal cord, lateral paragigantocellular nucleus, nucleus raphe magnus, locus coeruleus, periaqueductal grey, thalamus, and sensorimotor cortex were sampled with tissue corers of appropriate size. To minimize RNA breakdown, the tissues were kept on ice and homogenized in RNA extraction buffer within 7 min of sacrifice. Individual tissues of five to 10 rats were combined to form one sample.

A total of 12–15 consecutive coronal sections of the lumbosacral spinal cord were examined and the dorsal nuclear grey was sampled bilaterally with a 1-mm tissue corer. Bilateral samples of the lateral paragigantocellular nucleus were dissected (and combined) from three consecutive coronal sections of rat brain, approximated between −10.04 and −13.24 mm from bregma [31]. The most rostral of three sections was identified by the presence of the facial nerve. A 1-mm tissue corer was placed along the ventral edge of the medulla, adjacent to the pyramidal tract in the first two sections and bordering on the inferior olive nuclei in the most caudal third section. Nucleus raphe magnus was dissected with a 2-mm tissue corer from four coronal sections of rat brain, approximately between −9.16 and −13.24 mm from bregma [31], including three sections sampled for lateral paragigantocellular nucleus. The most rostral section was identified by the shape and size of the inferior colliculi. The corer was placed along the midline, dorsally to the nuclei of the inferior olive in the most caudal section, or to the

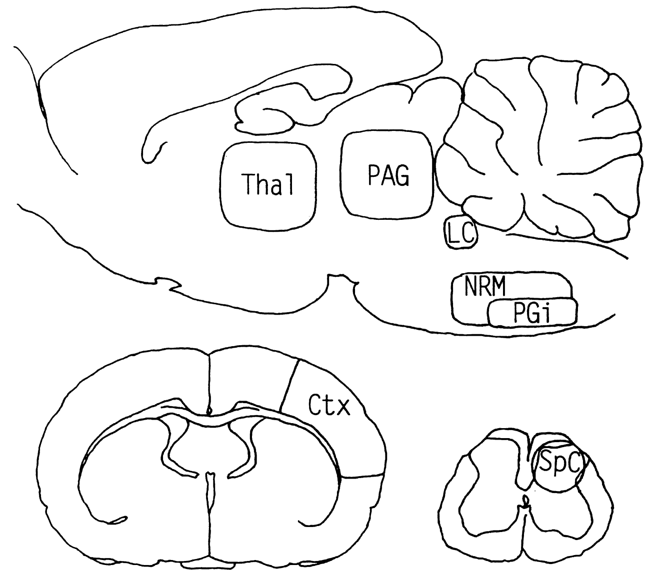

Fig. 1. A schematic drawing illustrating the seven regions of rat CNS selected for NR1 mRNA analysis. The regions of the lateral paragigantocellular nucleus (PGi), nucleus raphe magnus (NRM), locus coeruleus (LC), periaqueductal grey (PAG), medial thalamus (Thal) (depicted in sagittal view), sensorimotor cortex (Ctx) and spinal cord dorsal horn (SpC) (outlined on coronal sections), were identified according to the atlas of Paxinos and Watson [31]. The anterior and posterior boundaries of each region were based on the distance from bregma while the vertical dimensions (in the sagittal view) reflect the size of the tissue corers utilized to collect the samples. The tissues were dissected on ice within 7 min of sacrifice and immediately homogenized in RNA extraction buffer.

pyramidal tracts in the other three sections. Locus coeruleus was identified in one or two consecutive coronal sections approximately between −9.16 and −10.30 mm from bregma [31], as areas of nuclear grey located at the lateral extremes of the fourth ventricle. Bilateral samples were collected with a 1-mm tissue corer and combined. Periaqueductal grey was obtained with a 3-mm tissue corer, as the nuclear grey surrounding ventricular aqueduct in three consecutive sections, approximately −5.20 to −8.30 mm from bregma. Medial thalamus was dissected with a 3-mm tissue corer in three consecutive sections between −1.30 and −4.30 mm from bregma, and defined as the central region immediately ventral to the hippocampus. Bilateral samples of the sensorimotor cortex were dissected freehand from two consecutive sections immediately anterior to the hippocampus (0.70 to −1.3 mm from bregma). These regions were removed within 7 min of sacrifice and immediately homogenized in a buffer containing guanidinium isothiocyanate [15]. Individual tissues from five to 10 rats were pooled, and samples were collected in two to four separate micro-dissections.

Total cellular RNA was extracted with phenol and precipitated with ethanol according to a procedure that routinely yields RNA recoveries of 77.0±7.2% (S.D.) [15]. A total of 20 μg of glycogen (Boehringer-Mannheim, Germany) was used as a carrier during extraction.

2.6. mRNA analyses by solution hybridization

A ^{32}P-labelled riboprobe complementary to the NR1 mRNA (specific activity 6.6×10^{8} dpm/mg) was prepared using an SP6 transcription system, from a [pGEM-7zf(+)] plasmid containing the 1413-base-long sequence present in a *Sma*I-*Sac*I fragment [15] of pN60, a cDNA for the functional rat NMDA receptor subunit, NR1 [28]. This sequence codes for one of two putative glutamate binding sites and three of four transmembrane regions (TM1, TMII and TMIII) [28]. In the Northern blot, this riboprobe hybridizes to the two main forms of the NR1 mRNA, 4.4 and 4.2 kb in length, in RNA extracts from whole rat brain [15,28].

The details of a solution hybridization assay have been described elsewhere [15]. Briefly, total RNA extracts were incubated with 150 000 dpm of the MOR-1 riboprobe for 4 h at 75 °C, and then subjected to RNase digestion for 1 h at 30 °C. The RNase resistant duplexes which formed between the ^{32}P-labelled riboprobe and the complementary RNA were precipitated with trichloroacetic acid, collected on glass microfiber filter paper, and counted by liquid scintillation. A standard calibration curve was included in each assay to permit quantitation. The 4272 base long unlabelled sense transcript of the pN60 plasmid [28] served as the standard for NR1 mRNA quantitation. Total cellular RNA in each sample was quantified by solution hybridization, using rat liver RNA as the calibration standard and a ^{32}P-labelled riboprobe complementary to human 18S rRNA [15]. This allowed the levels of NR1 mRNA to be expressed as pg of mRNA per μg of total RNA. Duplicate measurements of the NR1 mRNA levels were performed in RNA extracts from tissue samples obtained in four separate micro-dissections (for spinal dorsal horn, nucleus raphe magnus, medial thalamus, and sensorimotor cortex) or in two separate microdissections (for paragigantocellular nucleus, locus coeruleus, and periaqueductal grey).

2.7. Membrane preparation and [^{3}H]MK-801 binding assay

For the binding saturation assays, whole brains and spinal cords were prepared from morphine or placebo treated rats as described by Gorman et al. [17]. The whole spinal cord was homogenized with a PT20 polytron in 50 mM Tris–acetate buffer (pH 7.4 at RT), then centrifuged (35 000×*g* for 15 min at 4 °C) twice. The pellets were resuspended in 50 mM Tris–acetate buffer, frozen in a methanol bath chilled with dry ice, thawed at RT and centrifuged again to remove excess endogenous excitatory amino acids. The pellets were resuspended in 50 mM Tris–acetate buffer, incubated at 37 °C for 20 min and centrifuged again (35 000×*g* for 20 min).

All binding assays were performed at 25 °C. [^{3}H]MK-801 (30 Ci/mmol, DuPont-NEN, Boston, MA, USA) was used to label total NMDA receptors. Six different concentrations of [^{3}H]MK-801 ranging from 1 to 20 nM were used. Nonspecific binding of [^{3}H]MK-801 to the NMDA receptors was determined in the presence of 100 μM unlabeled MK-801. Following 4 h or incubation at 25 °C, the samples were filtered through Whatman GF/B filters presoaked in 0.1% polyethylenimine for 1 h using a 24-well Brandel cell harvester. The filters were then washed twice with 5 ml cold Tris–HCl buffer and transferred to scintillation vials. All samples were incubated with scintillation cocktail overnight and then counted in a β-counter. Dissociation constants (K_d) and maximal number of binding sites (B_{max}) were determined [40,44].

2.8. Data analysis

All data were analyzed by the one-way analysis of variance (ANOVA) for multiple treatments followed by Newman–Keul's test if significant results were found or the *t*-test for paired treatment. Data from [^{3}H]MK-801 binding of each group were analyzed by the least square method for Scatchard analysis. The level of significance was $P<0.05$

3. Results

3.1. Co-administration of LY274614 attenuated morphine tolerance

There were no significant differences among the baseline hotplate latencies (Fig. 2), indicating that neither chronic LY274614 nor chronic LY274614 plus morphine altered the acute nociceptive response of the animal to the thermal stimulus employed in the hotplate test.

The morphine challenge significantly increased the hotplate latency in the sham-placebo group compared with baseline latency, demonstrating morphine's analgesic effects in morphine-naive animals. In contrast, the hotplate latency of the sham-morphine group on day 7 was not significantly different from their baseline latency, and was significantly shorter than the sham-placebo control group, indicating that the chronic morphine treatment paradigm rendered the rats tolerant to morphine's analgesic effect (Fig. 2).

Concurrent administration of LY274614 (24 mg/kg/24 h) via continuous s.c. infusion (LY274614-morphine group) attenuated the development of morphine tolerance. The challenge dose of morphine (10 mg/kg) increased the hotplate latency to the cut-off level in morphine-naive animals (Fig. 2). In contrast, the hotplate latency values of animals treated concurrently with LY274614 and morphine for 7 days (LY274614-morphine group) were significantly higher than those of morphine-treated animals that did not receive LY274614 (sham-morphine group) (Fig. 2). We have previously shown that chronic administration of

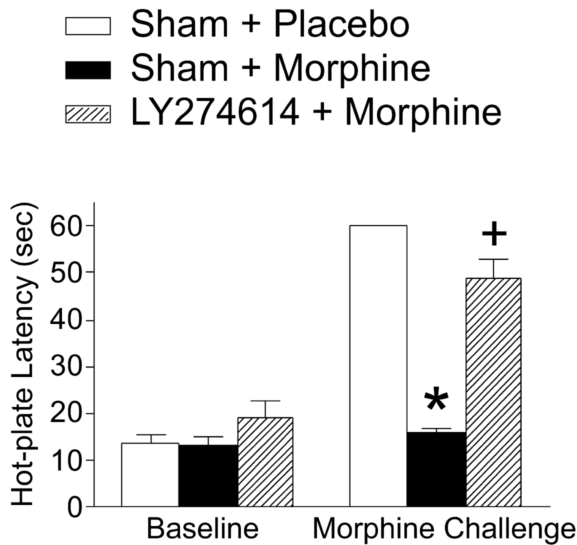

Fig. 2. Attenuation of morphine tolerance by LY274614 on treatment day 7. Rats received LY274614 (24 mg/kg/24 h) by continuous s.c. infusion via an implanted osmotic pump. Controls received sham surgery (sham). On day 1, 16 h after the pump implant or sham surgery, two wrapped morphine (Mor) pellets (75 mg base) or two placebo (Placebo) pellets were implanted s.c. On day 4, the animals were implanted with a second set of pellets. On day 7, the baseline (pre-injection) HP latency values were determined prior to the injection of morphine (10 mg/kg s.c) or saline challenge. The response HP latencies were assessed 60 min post-challenge. Following morphine challenge, the mean±S.E.M hotplate latencies of the sham-placebo and LY274614-morphine groups were significantly longer than the mean hotplate latencies of the sham-morphine group. * $P<0.05$ compared with placebo-implant control. + $P<0.05$ compared with sham-surgery control; $n=10$ per group.

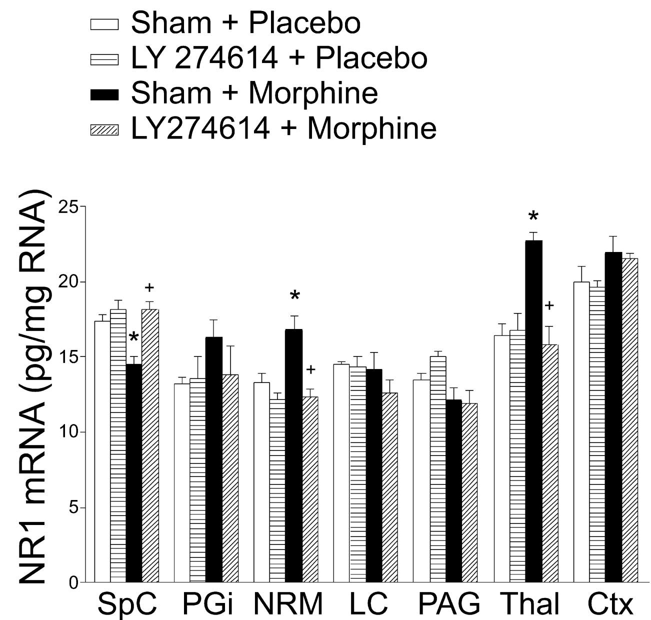

Fig. 3. The steady-state levels of NR1 mRNA (mean±S.E.M., expressed as pg/μg total RNA) in extracts from selected CNS regions as determined by solution hybridization. Chronic exposure to morphine resulted in a small (16%) but statistically significant decrease in the NR1 mRNA levels in SpC and an elevation in NRM and Thal (by 26 and 38% respectively) of the sham-morphine animals as compared to the sham-placebo controls. Concurrent administration of LY274614 prevented the changes in the levels of NR1 mRNA in SpC, NRM and Thal. The NR1 mRNA levels in these regions obtained from LY274614-morphine group were not significantly different from those of sham-placebo or LY274614-placebo controls. In the absence of morphine, the continuous infusion of LY274614 did not significantly alter the steady-state levels of NR1 mRNA compared to the sham-placebo controls. * $P<0.05$ compared with placebo-implant control. + $P<0.05$ compared with sham-surgery control.

LY274614 does not affect either the baseline hotplate latency or the response to a challenge dose of morphine [34,35]. Therefore, LY274614 affects the development of morphine tolerance and not its expression following the challenge dose.

3.2. Chronic morphine induced region specific changes in NR1 mRNA expression

In the sham-placebo controls, the mean steady-state NR1 mRNA levels in pg/μg RNA ranged from 13.2±0.5 in the paragigantocellular nucleus to 20.0±1.0 in sensorimotor cortex. Continuous morphine treatment produced region specific changes in NR1 mRNA expression in the rat CNS. Chronic morphine induced a small but significant decrease (16%) in the steady-state levels of NR1 mRNA in spinal cord dorsal horn and an elevation in nucleus raphe magnus and medial thalamus (by 26 and 38%, respectively) in the sham-morphine animals as compared to the sham-placebo controls (Fig. 3). Chronic morphine treatment did not alter the NR1 mRNA levels in the lateral paragigantocellular nucleus, locus coeruleus, periaqueductal grey, and sensorimotor cortex (Fig. 3).

With our experimental conditions, binding parameters for [^{3}H]MK-801 in cortex remained unchanged (Table 1). In contrast, in the spinal cord, K_d was not significantly altered, whereas B_{max} was significantly decreased to approximately 50% by chronic morphine treatment (Table 1), indicating a downregulation of the NMDA receptor in the spinal cord following chronic morphine treatment.

Table 1
[^{3}H]MK-801 binding parameters in brain and spinal cord homogenates from rats treated chronically with morphine or saline

	[^{3}H]MK-801 binding for NMDA receptors	
	Cortex	Spinal cord
K_d (nM)		
Saline	3.9±0.4	8.8±0.7
Morphine	4.4±0.7	11.3±0.7
B_{max} (fmol/mg tissue)		
Saline	2206±246	224±7.6
Morphine	2321±247	112±10.7*

Data are presented in mean±1 S.E.M. as determined by 2–4 binding assays for each group of rats.
* $P<0.05$ compared with the saline control group.

3.3. LY274614 prevented region specific changes in NR1 mRNA expression

Concurrent s.c. infusion of LY274614 with morphine prevented the changes in the NR1 mRNA levels in the spinal cord, nucleus raphe magnus and thalamus induced by chronic morphine (Fig. 3). The steady-state levels of NR1 mRNA were significantly higher in the spinal cord and lower in nucleus raphe magnus and thalamus in rats co-administered LY274614 and morphine (LY274614-morphine group) (Fig. 3). Furthermore, these levels in the LY274614-morphine animals were statistically not different from those of two control groups implanted with placebo pellets, sham-placebo and LY274614-placebo (Fig. 3).

In CNS regions where NR1 mRNA levels were not altered by chronic morphine treatment, chronic concurrent administration of LY274614 together with morphine did not significantly affect the expression of NR1 mRNA. These regions include lateral paragigantocellular nucleus, locus coeruleus, periaqueductal grey, and sensorimotor cortex (Fig. 3).

A 7-day infusion of LY274614 alone to the placebo group did not alter the steady-state NR1 mRNA levels in any of the regions studied, as compared to the sham-placebo group (Fig. 3).

3.4. Acute morphine treatment did not alter the levels of NR1 mRNA

In contrast to the region-specific changes of NR1 mRNA expression induced by chronic morphine treatment, an acute s.c. injection of morphine (10 mg/kg s.c.) to morphine-naive animals did not induce any significant changes in the NR1 mRNA levels in any of the CNS regions studied (Fig. 4).

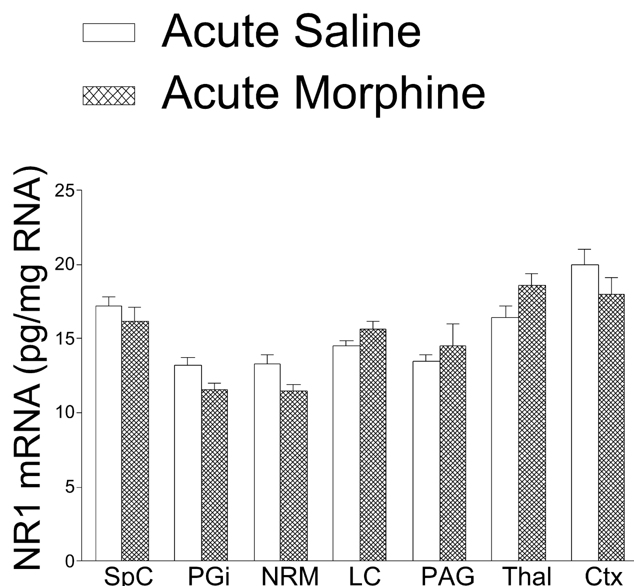

Fig. 4. Injection of a single dose of morphine (10 mg/kg s.c.) to morphine-naive animals did not significantly alter the steady-state levels of NR1 mRNA in any of the sampled CNS regions compared to the control animals that received a challenge injection of saline.

4. Discussion

The goals of the present study were first to define the effects of chronic morphine administration on the levels of mRNA coding for the NR1 subunit of the NMDA receptor complex in CNS regions associated with morphine tolerance and antinociception. Then we sought to determine the consequences of the co-administration of morphine and a dosage of LY274614 that can block the development of morphine tolerance [34,35] on the levels NR1 mRNA in these same CNS regions. Our data clearly demonstrated the region-specific changes in expression of the NMDA receptor NR1 subunit mRNA following chronic morphine treatment and the prevention of these changes by the competitive NMDA receptor antagonist LY274614 in the rat CNS. LY274614's effects were limited to the prevention of the changes in NR1 mRNA expression induced by chronic morphine since the chronic infusion of LY274614 alone did not result in changes of NR1 mRNA levels in any of the sampled regions.

The CNS regions selected for the study encompass areas that are rich in NMDA receptors [16,27,30,32,41] and contain high levels of the NR1 mRNA levels as determined by in situ and solution hybridization [15,28]. Furthermore, these regions are important in the transmission and modulation of nociception and in opioid antinociception, as these regions also exhibit medium to high densities of the μ-opioid receptors [33] and microinjection of morphine and other μ-opioid selective agonists into the spinal cord, nucleus raphe magnus, locus coeruleus, lateral paragigantocellular nucleus, periaqueductal grey and thalamus have been shown to produce naloxone-reversible antinociception [8,18,42]. Multiunit recordings from locus coeruleus, periaqueductal grey, and thalamus also indicate that these regions are sensitive to morphine [1,12]. Neuroanatomical connections among these regions have been extensively studied. The nuclei of medial thalamus receive projections from ascending nociceptive pathways (spinothalamic and paleospinothalamic tracts) and from brainstem structures associated with endogenous antinociceptive mechanisms, such as periaqueductal grey and nucleus raphe magnus [9,14,18]. Intralaminar thalamic nuclei in turn project to the sensorimotor cortex. Lateral paragigantocellular nucleus receives inputs from a wide variety of sources including periaqueductal grey and dorsal spinal cord [39] and is a major afferent to locus coeruleus. The brainstem structures are reciprocally connected with the dorsal horn of spinal cord [18].

The neuroanatomical approach described above, combined with a quantitative solution hybridization assay for the NR1 mRNA [15] allowed us to investigate the effect of chronic treatment with morphine alone or in combination with LY274614, on the levels of NR1 mRNA in selected regions of rat CNS. The morphine administration paradigm used in this study to render animals tolerant to the challenge dose of morphine has been shown to produce more than an 11-fold rightward shift in the morphine dose–response curve [34]. A concurrent s.c. infusion of LY274614 at a dose (24 mg/kg/24 h) blocks NMDA receptors [34,35] and prevents or reverses the development of analgesic tolerance to morphine [35].

Chronic morphine administration was found to produce significant changes in the mean steady-state levels of NR1 mRNA in the dorsal horn of the spinal cord, where a 16% decrease was detected, and in nucleus raphe magnus and medial thalamus (26 and 38% increase, respectively), but not in other regions analyzed. Our results in the spinal cord (Fig. 3 and Table 1) are in agreement with the downregulation of NMDA receptor binding reported by Bhargava et al. [4]. Also, our data agree with LeGreves et al. [23] in that a single dose of morphine does not affect NR1 mRNA levels (Fig. 4). We also find that the concurrent s.c. infusion of the NMDA antagonist LY274614 that attenuated the development of analgesic tolerance to morphine, also prevented or suppressed the changes in the mean steady-state levels of NR1 mRNA in the dorsal horn of spinal cord, nucleus raphe magnus and thalamus, induced by morphine treatment alone. This suggests that the region-specific changes in the expression of NR1 mRNA, induced by chronic morphine, are likely to reflect neuronal plasticity specifically associated with the development of tolerance, rather than with other effects of morphine administration.

The largest morphine-induced change in the steady-state NR1 mRNA levels was detected in the medial thalamus, which contains high levels of both μ-opioid and NMDA receptors [23,45]. Nucleus submedius, which lies within the sampled region of medial thalamus as it was defined in this study, receives a direct, virtually exclusive input from spinal and dorsal horn, suggesting a direct involvement in nociception. Clinical inferences confirm the role of medial thalamus in the modulation of nociception and the mechanisms of chronic pain [21]. The reuniens nucleus, which also lies within the sampled area, contains many neurons densely stained with the NR1 antibody, in addition to moderate overall immunostaining of all midline thalamic nuclei [32].

A 7-day infusion of LY274614 alone, at the dose of 24 mg/kg/24 h, does not alter either the animals' sensitivity to morphine assessed in the hot-plate test [34,35], or the levels of the NR1 mRNA in any of the CNS regions sampled in this study. Thus, the ability of LY274614 co-administration with morphine to suppress the changes induced by chronic morphine alone does not result from non-specific effects of treatment with a receptor antagonist on the expression of this receptor mRNA.

To verify that the changes in the mean steady-state levels of NR1 mRNA in selected CNS regions of tolerant animals result from the 7-day morphine treatment paradigm and not from the acute challenge with morphine [35], a separate group of sham-operated, placebo-implanted controls was challenged with morphine at 1.5 h prior to sacrifice. As expected, these animals were fully sensitive to morphine, reaching the 60 s hot-plate cut-off latency. No significant differences in the levels of the NR1 mRNA were found between the morphine- and saline-injected animals. It is also important to note that none of the treatment paradigms utilized in the study altered the subsequent ability of animals to respond to the HP test, since there were no significant differences in the base-line (pre-challenge) latencies among the four treatment groups.

The biochemical findings presented here indicate that alterations in the expression of NR1 mRNA in three CNS regions involved in transmission and modulation of nociception are associated with and/or may contribute to the development of tolerance. It remains to be determined whether the changes in the steady-state NR1 mRNA levels are due to altered rates of transcription or a change in mRNA stability. The range of changes reported here (16–38%) is comparable to the magnitude of up- or downregulation of other genes whose expression is altered in morphine tolerant animals [29,38]. In this study we also observed a 50% decrease in MK-801 binding in the spinal cord of animals exposed to the chronic morphine treatment paradigm (Table 1). The binding of MK-801, an open channel blocker of the NMDA receptor-ion channel complex, provides an estimate of the downregulation of the receptor complex consequent to chronic morphine and clearly demonstrates that, in this study changes in NR1 mRNA are associated with alterations in receptor protein. Since there is considerable amplification at the stage of mRNA translation, a relatively small change in mRNA availability can lead to pronounced alterations in the levels of receptor protein as was the case here.

Our results indicate that morphine tolerance is associated with a downregulation of spinal cord NMDA receptors and that conditions that prevent the development of morphine tolerance (i.e. the co-administration of an NMDA receptor antagonist) also prevent the downregulation of the receptor. Downregulation of spinal glutamate transporters occurs during the development of morphine tolerance and these changes in the transporters can be prevented by MK-801 [26]. Based on these observations the downregulation of spinal NMDA receptors appears to result from changes in glutamate homeostasis resulting from inhibition of the glutamate transporter by morphine. These transporters play an important role in the removal of glutamate from the synapse following release from the presynaptic terminal. Inhibition of transporter function can be expected, at least initially, to increase synaptic gluta-

mate and result in activation of the NMDA–NO cascade leading to the production of NO [7] and altered gene expression, such as induction of c-fos [3]. Furthermore, occupancy of the μ-receptor by morphine results in the activation of PKC [10]. PKC can phosphorylate the NMDA receptor, removing the Mg^{2+} blockade and further increasing calcium permeability of the receptor [11]. The enhanced glutamatergic activity in the spinal cord [26] during morphine treatment may result in a compensatory downregulation of NMDA receptors as we observed. The addition of an NMDA receptor antagonist may have prevented the decrease in NR1 expression by occupying the NMDA receptor and thus shielding it from the effect of increased glutamate availability. In addition, an NMDA receptor antagonist would be expected to reduce the NMDA receptor-mediated calcium influx and should therefore be able to prevent any changes in receptor expression resulting from increased intracellular calcium. Recently, mechanisms have been advanced to explain desensitization and/or downregulation of the μ-opioid receptor after morphine. These mechanisms include receptor oligomerization and endocytosis [2,6] and activation of PKC and β-arrestin [5,6,43]. A contribution to μ-receptor desensitization by PKC would appear to provide a sequence of events that begins with activation of the μ-opioid receptor by morphine followed by NMDA receptor activation by PKC, which allows the influx of calcium, which in turn activates additional calcium-dependent kinases, including PKC, ultimately resulting in the desensitization of the μ-opioid receptor. Concurrent with these events is the inhibition by morphine of the spinal glutamate transporters, which, at least initially, provides increased glutamatergic tone. Because the NMDA receptor mediated calcium influx also activates NO, a diffusible signaling molecule, the NMDA receptor mediated effects can be expected to influence both pre and postsynaptic μ-opioid receptors in the spinal cord.

While this mechanism can provide an explanation for our observations on the downregulation of spinal NMDA receptors, the role of glutamate transporters in morphine tolerance at supraspinal sites is not yet known.

The paradigm we demonstrate here provides a useful approach for optimizing the identification of biochemical changes that are directly associated with morphine tolerance.

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